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Differential Expression of Interleukin-17A and -17F Is Coupled to T Cell Receptor Signaling via Inducible T Cell Kinase

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SUMMARY

T helper 17 (Th17) cells play major roles in autoimmunity and bacterial infections, yet how T cell receptor (TCR) signaling affects Th17 cell differentiation is relatively unknown. We demonstrate that CD4$^+$ T cells lacking Itk, a tyrosine kinase required for full TCR-induced phospholipase C-γ (PLC-γ1) activation, exhibit decreased interleukin-17A (IL-17A) expression in vitro and in vivo, despite relatively normal expression of retinoic acid receptor-related orphan receptor-γT (ROR-γT) and IL-17F. IL-17A expression was rescued by pharmacologically induced Ca^{2+} influx or constitutively activated nuclear factor of activated T cells (NFAT). Conversely, decreased TCR stimulation or calcineurin inhibition preferentially reduced IL-17A expression. We further found that the promoter of *Il17a* but not *Il17f* has a conserved NFAT binding site that bound NFATc1 in wild-type but not Itk-deficient cells, even though both exhibited open chromatin conformations. Finally, *Itk*$^{-/-}$ mice also showed differential regulation of IL-17A and IL-17F in vivo. Our results suggest that Itk specifically couples TCR signaling to *Il17a* expression and the differential regulation of Th17 cell cytokines through NFATc1.

INTRODUCTION

One of the hallmarks of adaptive immune responses is the differentiation of CD4$^+$ T helper cells into distinct effector populations that are required for orchestrating responses to infection. First recognized and best studied are the T helper 1 (Th1) and Th2 cell subclasses, which produce interferon-γ (IFN-γ) and interleukin-4 (IL-4), respectively, and have distinct effector functions. However, it is now appreciated that there are multiple effector cell populations that can result from activation of naive CD4$^+$ T cells (Zhou et al., 2009; Zhu and Paul, 2008). One of these, the Th17 cell lineage, has recently been recognized for its major role in autoimmunity and responses to bacterial infections (Bettelli et al., 2007; Weaver et al., 2007). Th17 cells were first identified by their ability to produce IL-17A, a cytokine that helps recruit neutrophils and is important for driving inflammatory responses. In the mouse, Th17 cells differentiate in response to transforming growth factor-β1 (TGF-β1) and IL-6 (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006); additionally, IL-21 helps promote Th17 cell differentiation (Korn et al., 2007; Nurieva et al., 2007a; Wei et al., 2007; Zhou et al., 2007). These cytokines, via a pathway that requires signal transducer and activator of transcription-3 (STAT-3), turn on expression of the key transcription factors retinoic acid receptor-related orphan receptor-γT (ROR-γT) and RORα, which are critical for expression of *Il17a*, as well as the closely linked *Il17f* gene, and *Il21* and *Il22* (Ivanov et al., 2006; Yang et al., 2008b).

Many studies have helped elucidate the key role of cytokines in regulating lineage-specific transcription factors and the differentiation of distinct effector CD4$^+$ T cell populations (Zhu and Paul, 2008). However, in the Th1-Th2 paradigm, it is well established that signaling from the T cell receptor (TCR) also contributes to the development and establishment of cell fate. T cells need to be activated through the TCR in order to produce effector cytokines, and multiple lines of evidence, including the use of different antigen concentrations and altered peptide ligands, have demonstrated that varying conditions of TCR ligation induce differential patterns of cytokines both in vitro and in vivo (Constant and Bottomly, 1997). Furthermore, distinct components of TCR signaling have also been linked to differentiation or establishment of effector cell function, including proximal signaling components and, more distally, transcription factors (Glimcher and Murphy, 2000; Mowen and Glimcher, 2004). How TCR signaling affects IL-17 production is relatively unknown. Moreover, whether these signaling pathways contribute to the regulation of the distinct Th17 cell cytokines is unclear.

The Tec family tyrosine kinase inducible T cell kinase (Itk) is a critical modulator of TCR signaling, where it functions to regulate phospholipase C-γ (PLC-γ) activation, as well as actin polarization and cell adhesion (Berg et al., 2005). Mutations affecting Itk reduce TCR-induced PLC-γ phosphorylation and downstream Ca^{2+} mobilization—these defects are worsened by mutations affecting both Itk and the related resting lymphocyte kinase (Rlk, also known as Txk) (Berg et al., 2005; Liu et al., 1998; Schaeffer et al., 1999). Accordingly, cells from *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ mice

show decreased responses to TCR stimulation that are associated with impaired TCR-induced activation of the Ca^{2+}-sensitive nuclear factor of activated T cell (NFAT) transcription factors (Fowell et al., 1999; Liao and Littman, 1995; Schaeffer et al., 2001). However, unlike mutations affecting more proximal TCR signaling molecules, mutations affecting the Tec kinases do not completely prevent or eliminate TCR signals, but rather lead to graded defects in T cell function, permitting evaluation of T cell function under conditions of partial or impaired TCR signaling (Gomez-Rodriguez et al., 2007; Schwartzberg et al., 2005).

To evaluate how TCR signaling affects Th17 cell differentiation, we examined cytokine production by TCR-stimulated sorted naive $CD4^+$ T cells isolated from *Itk*$^{-/-}$, *Rlk*$^{-/-}$*Itk*$^{-/-}$, and wild-type (WT) mice. We find that *Itk*$^{-/-}$ cells showed reduced expression of IL-17A, but, surprisingly, expression of the other Th17 cell cytokines including the closely linked *Il17f* gene is relatively intact. Moreover, *Itk*$^{-/-}$ cells showed normal expression of the transcription factors RORγT and RORα. Although *Itk*$^{-/-}$ cells have reduced responses to IL-6, expression of IL-17A was only poorly rescued by expression of a constitutively activated STAT-3 construct. Instead, IL-17A expression could be rescued by treatment with a Ca^{2+} ionophore or expression of an activated mutant of NFATc1. Conversely, specific decreases in expression of IL-17A (yet relatively normal expression of IL-17F) could be recapitulated by either low-dose TCR stimulation or treatment with low-dose inhibition of Calcineurin. Consistent with these findings, we show that the region upstream of the promoter of *Il17a*, but not *Il17f*, has a NFAT binding site that is conserved across species and is occupied by NFATc1 in WT but not *Itk*$^{-/-}$ Th17 cells, despite both promoters having open chromatin conformations. Finally, *Itk*$^{-/-}$ mice also showed evidence for impaired production of IL-17A in vivo, despite relatively normal expression of IL-17F. Together, these data argue that the expression of IL-17A is specifically coupled to TCR signaling via Itk-mediated regulation of NFAT.

RESULTS

Itk$^{-/-}$ $CD4^+$ T Cells Show Defective IL-17A Production

To evaluate how TCR signaling affects Th17 cell differentiation, we examined intracellular cytokine production from sorted naive ($CD44^{lo}CD62L^{hi}$) $CD4^+$ T cells isolated from *Itk*$^{-/-}$, *Rlk*$^{-/-}$*Itk*$^{-/-}$, and WT mice that were stimulated with anti-CD3 plus anti-CD28 in the presence of WT antigen-presenting cells (APCs) and polarizing cytokines. Both *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ $CD4^+$ T cells were capable of differentiating into IFN-γ-producing cells under Th1 cell differentiation conditions (IL-12 plus anti-IL-4), although at slightly lower percentages than WT cells (Figure 1A). In contrast, after exposure to TGF-β1 and IL-6, potent inducers of Th17 cell differentiation, there were marked reductions in the percentage of cells that had differentiated into IL-17A-producing cells from both *Itk*$^{-/-}$ (16.5% ± 1.9%) and *Rlk*$^{-/-}$*Itk*$^{-/-}$ (11% ± 1.9%) animals as compared to WT cells (50.4% ± 4.0%) (Figure 1A). Similar results were seen with $CD8^+$ T cells, which can also differentiate to produce IL-17A under these conditions (data not shown). Similar to a recent report (Veldhoen et al., 2009), we found that growth in the Iscove's modified Dulbecco's media (IMDM), which contains higher amounts of aryl hydrocarbons, improved Th17 cell differentiation (Figure 1A, data points in red). However, *Itk*$^{-/-}$ cells were defective in differentiation to IL-17A-producing cells in either media. To focus our evaluation on the effects of Itk and TCR signaling during the differentiation to IL-17A-producing cells, we restimulated cells with PMA and ionomycin in these studies. However, the defects in IL-17A expression were even more severe when $CD4^+$ T cells were restimulated with anti-CD3 plus anti-CD28 (Figure 1B), arguing that *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ cells show defects both in priming to IL-17A-producing cells and in TCR-induced expression of IL-17A.

Itk$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ $CD4^+$ T cells have defects in TCR-induced proliferation (Berg et al., 2005; Liao and Littman, 1995; Schaeffer et al., 1999), which can be severe in the case of *Rlk*$^{-/-}$*Itk*$^{-/-}$ T cells (cell yields were less than 10% those of WT cells under Th17 cell culture conditions; see Figure 1A). To evaluate the possibility that decreased IL-17A production resulted from poor proliferation, we stained cells with carboxyfluorescein succinimidyl ester (CFSE) to follow cell division. Although *Itk*$^{-/-}$ cells did exhibit reduced cell division, decreased percentages of cells producing IL-17A were observed at each division compared to WT cells (Figure 1C and Figure S1, available online). These results suggested that the defect in IL-17A expression in *Itk*$^{-/-}$ cells did not result solely from impaired cell division and reflected an actual defect in IL-17A production. However, to minimize the effects of decreased cell proliferation, we focused our further studies on *Itk*$^{-/-}$ rather than *Rlk*$^{-/-}$*Itk*$^{-/-}$ cells.

Previous studies have shown that Itk deficiency alters thymic development and selection so that a large number of innate-type memory phenotype $CD8^+$ T cells develop in *Itk*$^{-/-}$ mice (Berg, 2007). To rule out the possibility that altered development contributes to the reduced production of IL-17A, we sorted naive WT and *Itk*$^{-/-}$ $CD4^+$ T cells, activated them under null conditions with blocking cytokine antibodies, and then retrovirally transduced them with a retrovirus expressing murine *Itk* for 1 day prior to exposing them to Th17 cell-inducing cytokines. Re-expression of Itk completely rescued the defect in IL-17A production in *Itk*$^{-/-}$ cells during Th17 cell differentiation (Figure 1D). Thus, efficient production of IL-17A requires Itk at the time of differentiation and does not appear to result from altered development.

Decreased IL-17A Message in Itk-Deficient Cells

To evaluate potential mechanisms for the decreased IL-17A production, we examined *Il17a* mRNA expression by quantitative-RT-PCR (q-RT-PCR) after 3.5 days of stimulation in RPMI media. *Il17a* message was markedly decreased in *Itk*$^{-/-}$ cells, demonstrating that the reduction in IL-17A production occurred at the level of *Il17a* mRNA (Figure 2A). Surprisingly, however, expression of the message of other Th17 cell cytokines, including *Il22* and *Il21*, appeared normal at this time of analysis. Indeed, expression of the closely linked *Il17f* gene was relatively intact, whereas expression of *Il17a* was consistently reduced at all times examined from 24 to 84 hr after stimulation (Figure 2A and data not shown). The differential effects on IL-17A and IL-17F were further evaluated by intracellular staining for cytokine production and ELISA for secreted cytokines (Figures 2B–2D). Although intracellular staining did reveal statistically significant reductions in IL-17F production (Figure 2C, $p < 0.001$), the difference was much less than seen for IL-17A (*Itk*$^{-/-}$ 82% WT values for IL-17F, versus 32% for IL-17A) and may reflect other defects

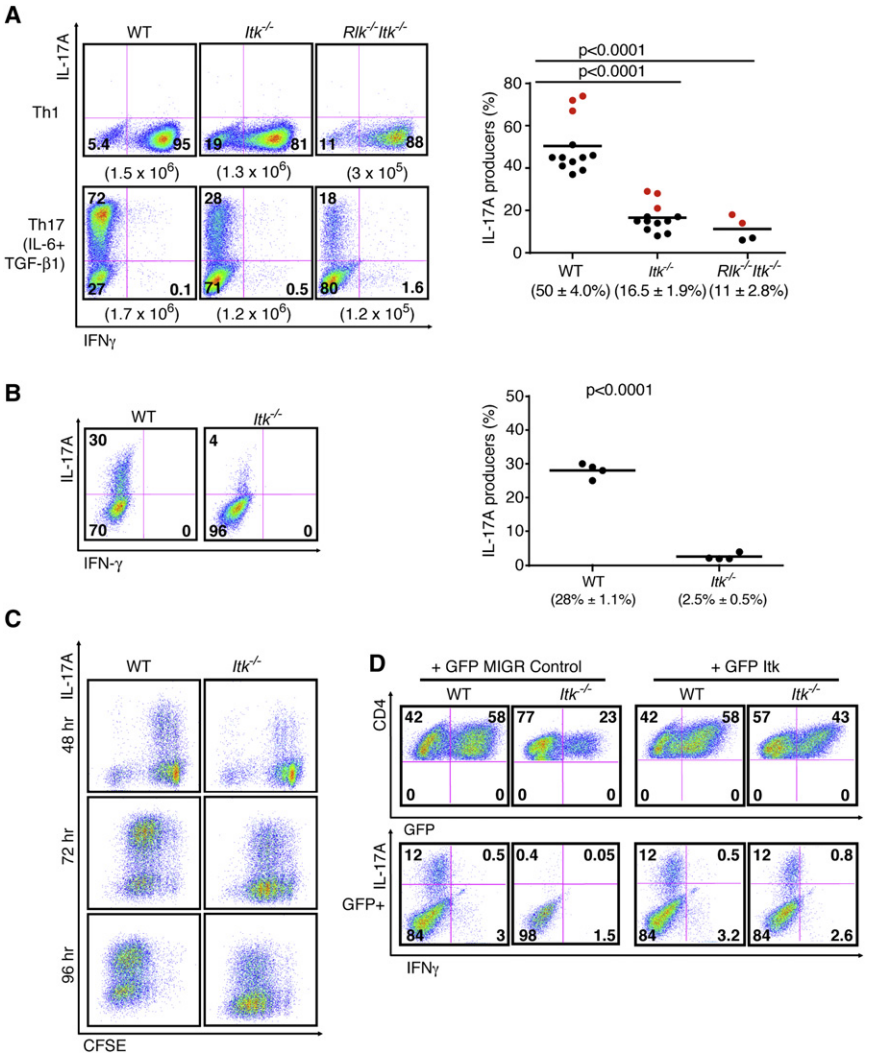


Figure 1. Reduced IL-17A Production from *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ CD4$^+$ T Cells

(A) Naive (CD62L^{hi}CD44^{lo}) CD4$^+$ T cells were stimulated with 1 μg/ml anti-CD3 and 3 μg/ml CD28 in the presence of WT T depleted splenocytes under either Th1 cell- or Th17 cell-inducing conditions for 3.5 days, restimulated with PMA and ionomycin for 4 hr and cytokine production analyzed by intracellular staining. Cell yields at end of culture are indicated in parentheses below each flow plot. Data are representative of four experiments for *Rlk*$^{-/-}$*Itk*$^{-/-}$ cells and over ten for *Itk*$^{-/-}$ cells. The right panel shows percentages of IL-17A CD4$^+$ T cell producers from individual experiments after differentiation for 2–3 days (experiments in IMDM indicated in red). Statistics were calculated with paired Student's t test.

(B) Naive (CD62L^{hi}CD44^{lo}CD25$^-$) CD4$^+$ T cells were stimulated as above, but restimulated with anti-CD3 plus anti-CD28 prior to intracellular cytokine staining. The right panel shows scatterplots of IL-17A CD4$^+$ T cell producers from four experiments.

(C) Naive CD4$^+$ T cells were labeled with CFSE and then differentiated and stained as above. For overlay, see Figure S1.

(D) Naive CD4$^+$ T cells were stimulated for 2 days under Th0 conditions, then infected with a control (MIGR) or MIGR-Itk-expressing retroviral vector for 24 hr prior to exposure to Th17 cell-inducing cytokines. Two days later, cells were restimulated with PMA and ionomycin and stained for expression of IL-17A and IFN-γ.

in *Itk*$^{-/-}$ cells that, for example, may affect protein translation. Similar results were seen with secreted cytokines as evaluated by ELISA (Figure 2D), where the differences in IL-17A secretion between WT and *Itk*$^{-/-}$ were much greater than the differences in IL-17F secretion. These results suggested that there may be distinct features of the regulation of the individual Th17 cell cytokines.

Itk$^{-/-}$ Mice Show Impaired Production of IL-17A In Vivo

Evaluation of allergic asthma responses have suggested that IL-17A contributes to pathology whereas IL-17F may be protective. Mice deficient in IL-17F show exacerbated responses associated with increased Th2 cell cytokine production in a model of allergic asthma, whereas mice deficient in IL-17A show decreased responses (Yang et al., 2008a). Interestingly, *Itk*$^{-/-}$ mice also have decreased responses to allergic asthma that have previously been associated with reduced Th2 cell cytokine production and T cell infiltrates (Ferrara et al., 2006; Mueller and August, 2003). To determine whether defects in IL-17A production were also observed in vivo, we examined responses in a model of allergic asthma (Figure 3A). Evaluation of IL-17 expression confirmed that *Il17a* mRNA was more severely reduced than *Il17f* in lungs of challenged *Itk*$^{-/-}$ mice, even when normalized for decreased numbers of T cells (Figure 3B). Thus, T cells from *Itk*$^{-/-}$ mice showed decreased expression of *Il17a* despite relatively normal *Il17f* expression in vivo, as well as in vitro.

Altered Responses to Cytokines

To further understand the nature of the defect in IL-17A production in *Itk*$^{-/-}$ cells, we evaluated responses to different cytokine milieus. Defects in IL-17A production were observed in response to multiple cytokines, including IL-21 plus TGF-β1, IL-1 plus IL-6, TGF-β1 plus IL-1, and TGF-β1 plus IL-6 and IL-23, suggesting that the decrease in IL-17A production was a universal defect that occurred in response to many, if not all, Th17 cell-inducing cytokines conditions (Figure 4A, Figure S2, and data not shown).

We next evaluated the response of *Itk*$^{-/-}$ cells to individual cytokines. Exposure of CD4$^+$ T cells to TGF-β1 induces their differentiation to Forkhead box P3 (FoxP3)-expressing induced T regulatory (iTreg) cells, especially in the context of IL-2 (Chen et al., 2003; Davidson et al., 2007). *Itk*$^{-/-}$ CD4$^+$ T cells were able to differentiate normally to FoxP3-expressing cells under these conditions, suggesting that responses to TGF-β1 were normal (Figure 4B). In contrast, whereas exposure of WT CD4$^+$ T cells to IL-6 gave rise to a small percentage of IL-17A-producing

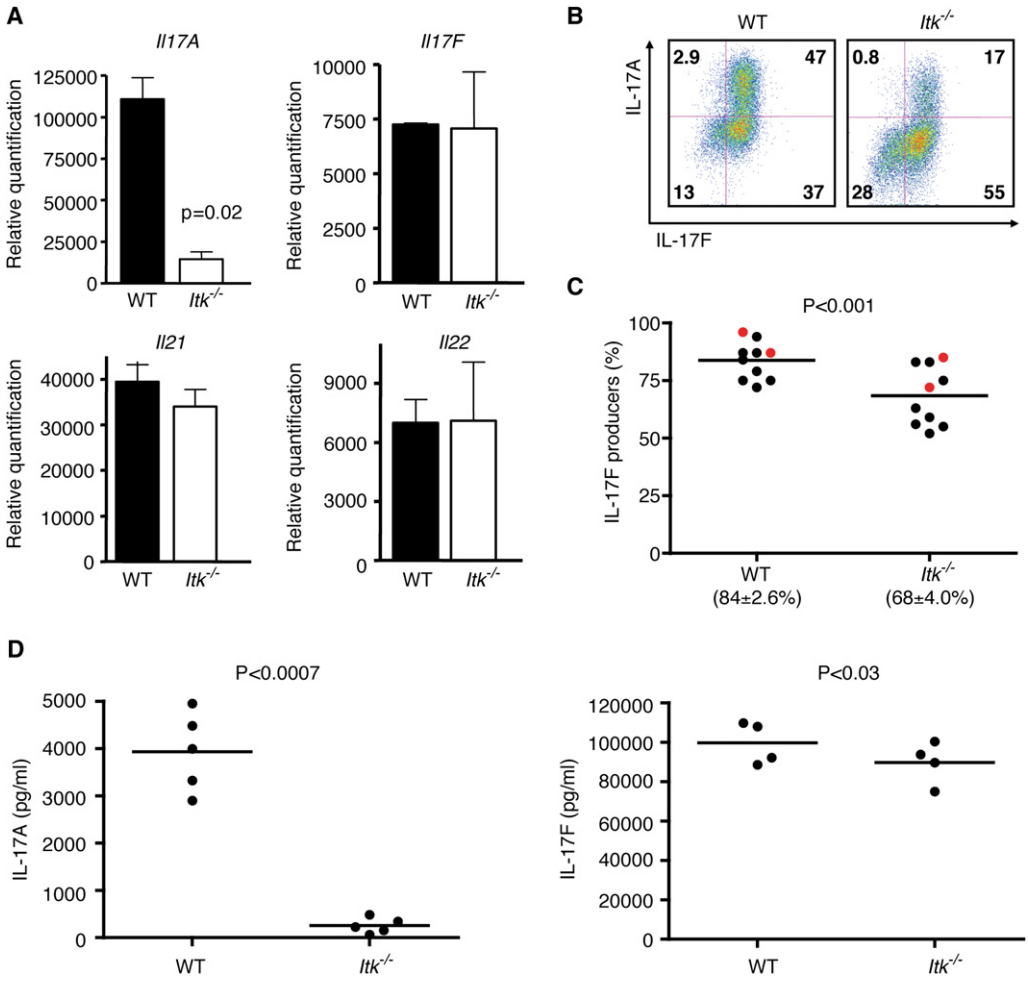


Figure 2. Itk Is Required for Efficient Transcription of *Il17a*, but Not *Il17f*

(A) Naive CD4$^+$ T cells were differentiated under Th17 cell conditions, and mRNA was analyzed by qRT-PCR after 3.5 days (84 hr) of differentiation (mean of 5 experiments ± SEM). Statistics were calculated with the paired Student's t test. Similar results were seen at 48 hr.

(B) Intracellular staining for IL-17A and IL-17F of cells differentiated for 2 days under Th17 cell conditions.

(C) Scatterplots of total IL-17F CD4$^+$ T cell producers (single IL-17F plus double IL-17F plus IL17-A producers) from the subset of experiments, shown in Figure 1A, where IL-17F was measured. Experiments in IMDM are indicated in red.

(D) IL-17A and IL-17F secreted by WT and *Itk*$^{-/-}$ CD4$^+$ T cells differentiated for 48 hr, as determined by ELISA (IL-17A: WT 3940 ± 374 versus *Itk*$^{-/-}$ 256 ± 73 pg/ml; IL-17F: WT 99700 ± 5400 versus *Itk*$^{-/-}$ 89700 ± 5390 pg/ml). Statistics were calculated with paired Student's t test.

cells (presumably because of low amounts of TGF-β1 in the serum or produced by cells in the culture because this differentiation could be blocked by anti-TGF-β), *Itk*$^{-/-}$ cells completely failed to differentiate into IL-17A-producing cells under these conditions (Figure 4A). In this experiment, *Itk*$^{-/-}$ CD4$^+$ T cells also produced lower amounts of IFN-γ in response to IL-6 in the presence of anti-TGF-β. These results suggest that Itk-deficient cells do not respond optimally to IL-6 upon activation.

IL-6 activates STAT-3, a transcription factor that is required for expression of RORγT, a key transcription factor required for IL-17A production (Ivanov et al., 2006; Yang et al., 2007). Expression of an activated STAT-3 mutant can drive activated T cells to express RORγT and produce more IL-17A (Yang et al., 2007). To evaluate whether loss of Itk affected STAT-3, we examined STAT-3 phosphorylation, which is required for its activation. In response to IL-6, either alone or under Th17 cell stimulation conditions (in combination with TGF-β1), Itk-deficient T cells exhibited variable decreases in STAT-3 phosphorylation (Figure 4C). Nonetheless, we observed normal *Rorc* and *Rora* mRNA in *Itk*$^{-/-}$ cells, suggesting that this decreased phosphorylation of STAT-3 was not sufficient to affect expression of these transcription factors (Figure 4D). Although we did observe early decreases in the expression of *IL-21*, which is also induced by IL-6 (Korn et al., 2007; Nurieva et al., 2007a; Wei et al., 2007; Zhou et al., 2007), these delays were only transient (data not shown). Moreover, whereas retroviral transduction of cells with an activated STAT-3 mutant increased expression of IL-17A in WT cells, it only minimally rescued IL-17A expression in Itk-deficient cells (Figure 4E). Thus, altered STAT-3 activation did not appear to be the major cause of defective IL-17A production in *Itk*$^{-/-}$ cells, suggesting that Itk helps regulate IL-17A expression by a different or additional mechanism.

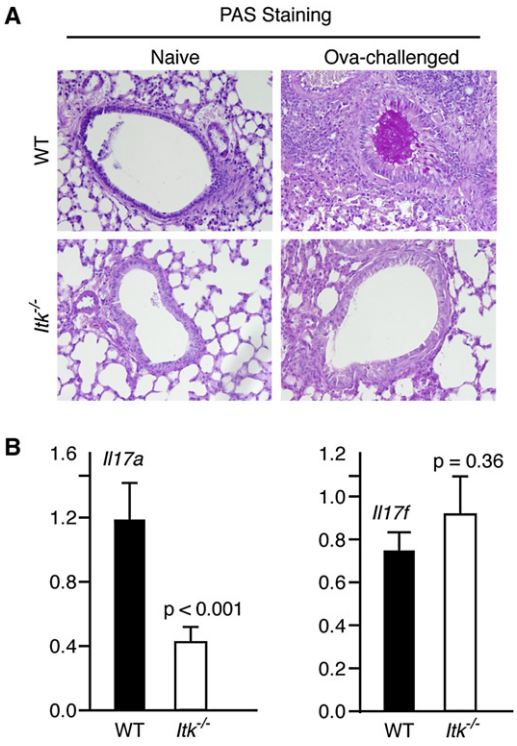


Figure 3. *Itk*$^{-/-}$ Mice Show Impaired Production of *Il17a* In Vivo

(A) Lung sections (PAS stained) from naive and OVA-challenged WT and *Itk*$^{-/-}$ mice.

(B) Expression of *Il17a* and *Il17f* mRNA in lungs from immunized and OVA-challenged WT and *Itk*$^{-/-}$ mice.

Data were expressed as $2^{-\Delta\Delta CT}$ relative to a treated WT mouse. *Thy1* was used for normalization of T cell numbers. Means ± SEM from 12 mice from four independent experiments are shown. Statistics were calculated with the paired Student's t test.

TCR Signaling Affects IL-17A Production

Itk is required for full TCR-induced activation of PLC-γ and downstream Ca^{2+} pathways (Berg et al., 2005). To evaluate whether TCR signaling affects IL-17A expression, we differentiated WT $CD4^+$ T cells in the presence of decreasing amounts of anti-CD3. We found that optimal production of IL-17A required high-dose anti-CD3 stimulation (Figure 5A). Intriguingly, lowering the dose of anti-CD3 stimulation preferentially affected the expression of IL-17A over IL-17F, similar to what we observed in *Itk*$^{-/-}$ cells. To determine whether altered TCR signaling contributes to the effects we see in *Itk*$^{-/-}$ cells, we differentiated cells with anti-CD3 in the presence of ionomycin, a Ca^{2+} ionophore that can rescue TCR-induced Ca^{2+} influx in *Itk*$^{-/-}$ cells (Liu et al., 1998). Stimulation with anti-CD3 plus ionomycin completely rescued IL-17A production in Th17 cell-polarized *Itk*$^{-/-}$ $CD4^+$ T cells, whereas expression of IL-17F was only minimally affected (Figure 5B). In contrast, stimulation with anti-CD3 plus PMA, which rescues ERK activation in *Itk*$^{-/-}$ T cells, did not rescue IL-17A production (data not shown). These results suggest that defects in TCR-induced Ca^{2+} mobilization contribute to the specific defect in IL-17A production in *Itk*$^{-/-}$ cells.

A Role for NFATc1 in Regulation of IL-17A

In T cells, Ca^{2+} signaling regulates activation and expression of a critical series of transcription factors, the NFATs, which are dephosphorylated by calcineurin, leading to their nuclear localization and activation (Gwack et al., 2007; Winslow et al., 2003). TCR stimulation in conjunction with costimulation further upregulates expression of NFATc1, which autoregulates its own expression (Nurieva et al., 2007b). We and others have previously shown that *Itk*$^{-/-}$ T cells exhibit defects in NFAT activation and nuclear localization, as well as the induction of NFATc1 expression observed upon activation with TCR and costimulation (Fowell et al., 1999; Nurieva et al., 2007b; Schaeffer et al., 2001). To evaluate whether NFAT activation affects IL-17A expression, we differentiated cells under Th17 cell conditions in the presence of increasing amounts of the calcineurin inhibitors FK506 or Cyclosporin A (CsA), which affect NFAT activation. Although treatment of cells with high amounts of FK506 affected both IL-17A and IL-17F (data not shown), exposure of cells to low amounts of FK506 preferentially affected expression of IL-17A (Figure 5C). Similar results were obtained with CsA (Figure S3). Thus, the extent of calcineurin inhibition appears to specifically affect expression of IL-17A compared to IL-17F.

To specifically evaluate the role of NFAT in regulating expression of IL-17, we examined the regions 20 kb upstream of the *Il17a* and *Il17f* transcriptional start sites, as well as the entire genes for potential NFAT binding sites. Although both promoters showed multiple potential NFAT consensus binding sites in the mouse, only the *Il17a* upstream region contained an NFAT binding site that was conserved across species (located 3085 bp upstream of the first exon, Figure 6A and Figure S4A). Evaluation of mouse *Il17a* promoter-luciferase constructs in the Jurkat T cell line revealed that expression of a 3.5 kb construct that included the potential NFAT binding site was specifically increased by coexpression of a constitutively active NFATc1 mutant (Figure 6B). This mutant is constitutively localized to the nucleus because of mutations affecting the negative-regulatory phosphorylation sites and does not require TCR activation for its nuclear localization (Neal and Clipstone, 2003). However, expression of activated NFATc1 did not affect expression of a similar-length construct derived from the *Il17f* promoter (Figure S4B). Furthermore, deletion of the conserved putative NFAT binding site in the *Il17a* promoter prevented the NFATc1-induced increase in luciferase activity (Figure 6B), suggesting that this site was a bona fide NFAT binding site.

To evaluate whether this NFAT binding site was used in vivo, we performed chromatin immunoprecipitation (ChIP) studies by amplifying this region after crosslinking and immunoprecipitating NFATc1, a major NFAT member expressed in mature T cells. ChIP analyses of WT cells differentiated under Th17 cell conditions demonstrated a large enrichment of NFATc1 binding to the conserved NFAT binding site in the *Il17a* promoter (Figure 6C). However, we saw no enrichment of amplification in samples from *Itk*$^{-/-}$ cells. Nonetheless, both WT and *Itk*$^{-/-}$ cells did show binding of acetylated histone H3 as well as K4-trimethylated histone H3 to the proximal promoter region upstream of the transcriptional start sites of both *Il17a* and *Il17f* genes, as well as to multiple conserved noncoding regions (CNS) in this locus (Figure S4C, Figure 7A, and data not shown). These results suggest that the entire *Il17* gene locus had undergone epigenetic modification consistent with open chromatin in both WT and *Itk*$^{-/-}$ cells. Thus, *Itk*$^{-/-}$ cells show a selective defect

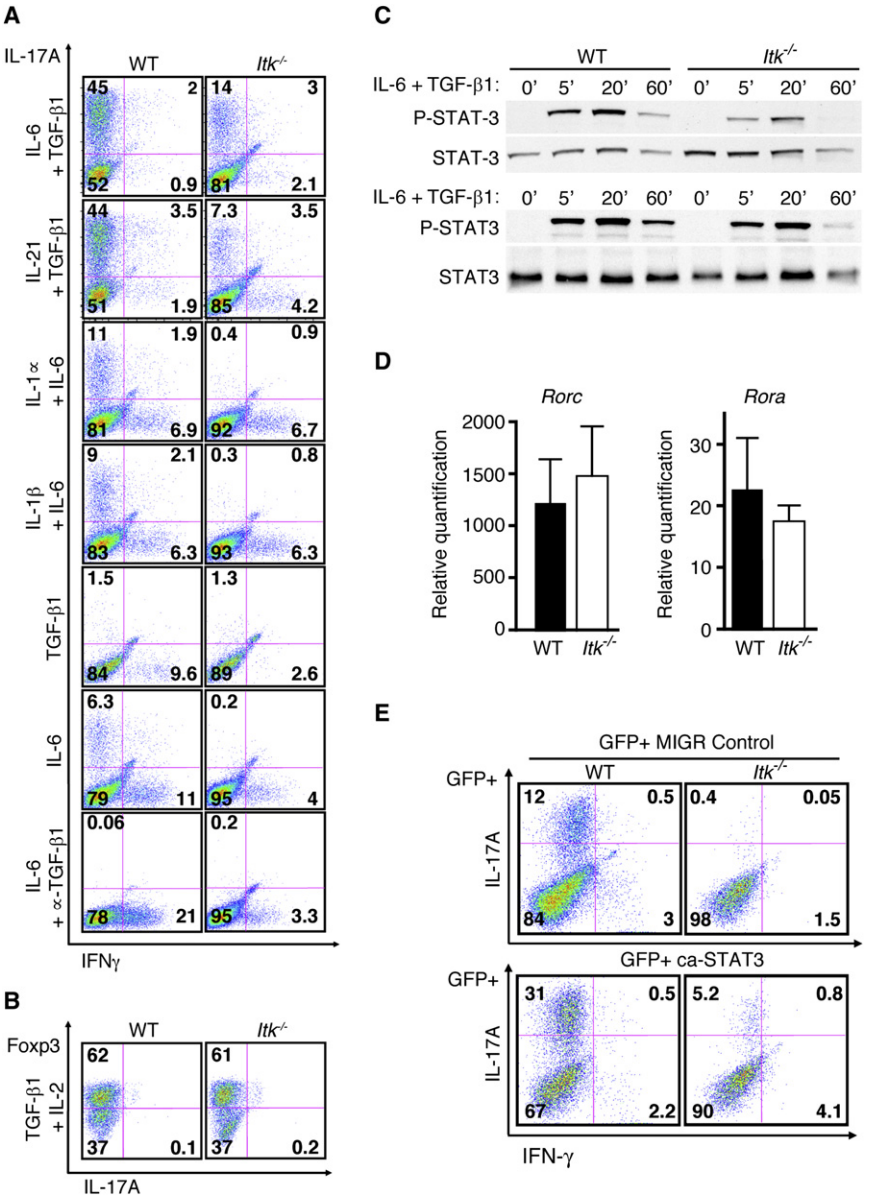


Figure 4. IL-17A Production in *Itk*$^{-/-}$ CD4$^+$ T Cells Is Reduced in Response to Multiple Cytokines, despite Normal Expression of *Rorc* and *Rora*

(A and B) Naive CD4$^+$ T cells were differentiated in the presence of the indicated cytokines (plus anti-IL-4, anti-IL-12, and anti-IFN-γ), and cytokine production was evaluated by intracellular staining for IL-17A plus IFN-γ (A) or IL17-A plus Foxp3 (B). Data are representative of over three experiments.

(C) T cells were stimulated with IL-6 plus TGF-β1 for the indicated times, lysed, and immunoblotted with anti-phospho-STAT3. Lower panels show total STAT3 levels. Two examples show the range of STAT-3 phosphorylation across six experiments.

(D) Naive CD4$^+$ T cells were differentiated under Th17 cell conditions for 84 hr and examined for *Rorc* and *Rora* mRNA by qRT-PCR (mean of five experiments ± SEM). Similar results were seen at 48 hr.

(E) IL-17A production is poorly rescued in *Itk*$^{-/-}$ T cells by expression of constitutively active STAT3. CD4$^+$ T cells were transduced with the indicated retroviral vector, and GFP$^+$ cells were examined for cytokine production after stimulation under Th17 cell conditions. Results are from the same experiment as Figure 1D for comparative purposes but are representative of 2–3 independent retroviral transduction experiments.

in the binding of NFATc1 to the *Il17a* promoter despite having an open chromatin conformation.

Finally, to determine whether the defect in NFATc1 activation causes the impaired *Il17a* transcription in *Itk*$^{-/-}$ cells, we transduced cells with a retroviral construct expressing activated NFATc1 (Neal and Clipstone, 2003). Transduction of the activated NFATc1 construct rescued IL-17A production in *Itk*$^{-/-}$ CD4$^+$ T cells, supporting the idea that defective NFAT activation is the cause of the decreased *Il17a* mRNA in these cells (Figure 7B).

DISCUSSION

Our results suggest that Itk specifically couples effective TCR signaling to the expression of IL-17A through its effects on the activation and expression of NFATc1. This defect in IL-17A expression is seen in response to multiple cytokines and is not the result of altered development given that it can be rescued by re-expression of Itk in preactivated cells. Our results agree with recent reports showing that high-dose CsA blocks IL-17A expression (Liu et al., 2005; Zhang et al., 2008a) and that interference with NFAT DNA binding activity can affect IL-17A production (Hermann-Kleiter et al., 2008). However, we demonstrate here that, interestingly, expression of other Th17 cell cytokines, including the closely linked *Il17f* gene, is not affected to the same extent as *Il17a* in *Itk*$^{-/-}$ T cells. Consistent with these observations, we find that low-dose FK506 or CsA preferentially affects IL-17A and that there is a conserved NFAT binding site upstream of the *Il17a* promoter, but not in the region 20 kb upstream of the *Il17f* gene. These results suggest that expression of *Il17a* is particularly sensitive to the strength of TCR signaling, requiring full activation of Ca^{2+}-mediated pathways, in addition to signals from cytokines required for the induction and activation of RORγT and STAT3. Consistent with this idea, we find that optimal expression of IL-17A requires high doses of anti-CD3 plus anti-CD28 or high-dose antigen stimulation (data not shown). Whether there are other factors that more specifically affect IL-17F production remains an interesting question. Intriguingly, sequence analyses demonstrate a conserved NF-κB binding site upstream of the start of the *Il17f* gene. Consistent with our data that the absence of Itk less severely affects IL-17F expression, we have previously found that activation of *Itk*$^{-/-}$ CD4$^+$ T cells in the presence of APCs has only modest effects on NF-κB activation (Schaeffer

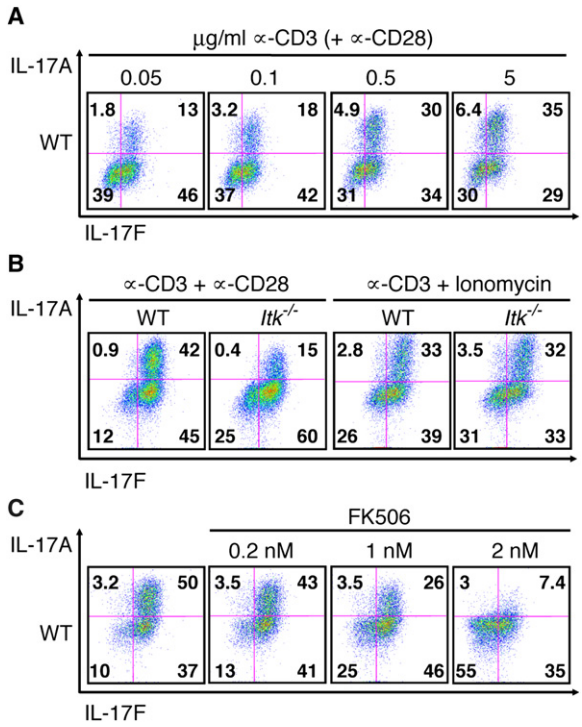


Figure 5. IL-17A Production Is Affected by TCR and NFAT Activation

(A) WT naive CD4$^+$ T cells were differentiated in the presence of varying amounts of anti-CD3 (plus 3 μg/ml anti-CD28) and stained for IL-17A and IL-17F.

(B) WT and *Itk*$^{-/-}$ naive CD4$^+$ T cells were differentiated with anti-CD3 plus anti-CD28 or anti-CD3 plus ionomycin and stained for cytokine production.

(C) WT naive CD4$^+$ T cells were differentiated with anti-CD3 plus anti-CD28, in the absence or presence of increasing amounts of FK506.

(A)–(C) represent three or more experiments, with varying concentrations of antibodies and inhibitors.

et al., 2001). We also note that high doses of FK506 or CsA can affect IL-17F production (although always to a lesser degree than IL-17A); however, these effects could result from secondary effects of NFATs or these inhibitors on expression of other genes, cell proliferation, or cell viability.

Recent data demonstrate that in addition to RORγT (Ivanov et al., 2006), RORα (Yang et al., 2008b), and interferon-regulatory factor 4 (Brustle et al., 2007), Runt-related transcription factor 1 (Runx1) also participates in the regulation of *Il17a* via a complex that can affect RORγT inhibition by FoxP3 (Zhang et al., 2008b). NFATs have been shown to couple with other transcription factors to differentially affect T cell functional outcomes (Hermann-Kleiter et al., 2008; Hu et al., 2007). Whether NFAT family members affect transcription of *Il17a* through secondary effects on these other transcription factors will be of interest. Our observation that multiple CNS in the *Il17* locus bind acetylated Histone H3 in *Itk*$^{-/-}$ T cells suggests that the *Il17* locus is in an open chromatin conformation, which would be consistent with the proper expression and activation of these other transcription factors (Kouzarides, 2007). Indeed, we see normal expression of RORγT, RORα, IRF4, and Runx1 upon differentiation of cells from *Itk*$^{-/-}$ mice (this paper and unpublished data). Nonetheless, we cannot rule out other contributions—although constitutively activated NFATc1 greatly improves IL-17A production in *Itk*$^{-/-}$ CD4 cells, other factors may also participate in pathways affecting IL-17A expression in these cells. For example, although we have not seen effects on SOCS3 expression (unpublished data), SOCS3 is known to affect STAT3 phosphorylation and Th17 cell differentiation (Chen et al., 2006). However, given the mild decreases in STAT3 phosphorylation, normal expression of *Rorc* and *Rora*, and minimal rescue with activated STAT3 constructs that we observed, it is likely that the major effects we see on IL-17A production result from effects on NFAT.

Why IL-17A specifically requires maximal TCR signaling for its expression is not clear but could be related to the functions of IL-17A, which is a powerful mediator of inflammation, strongly inducing inflammatory chemokines and neutrophil recruitment (Dong, 2008; Ouyang et al., 2008). Indeed, recent comparisons of IL-17A and IL-17F function indicated that IL-17A played a distinct and more critical role in the induction of certain inflammatory and autoimmune responses (Ishigame et al., 2009; Yang et al., 2008a). Intriguingly, the responses of *Itk*$^{-/-}$ mice in a model of allergic asthma resemble those of mice deficient in IL-17A, but not IL-17F (Mueller and August, 2003). Consistent with this observation, we find that *Il17a* expression is also decreased in lungs from *Itk*$^{-/-}$ mice relative to WT mice, even when we normalize to T cell numbers. Perhaps a secondary requirement for strong TCR signals provides a safety check to help regulate IL-17A responses, which although important for protection against bacterial infections, particularly in the gut, can also lead to pathological effects and more strongly promote autoimmunity than IL-17F.

These data support the idea that there may be subpopulations of Th17 cells that produce different cytokine patterns, which may be distinctly regulated. Indeed, regulation of IL-22, another Th17 cell cytokine, appears to be particularly sensitive to aryl hydrocarbons (Veldhoen et al., 2008). It is also of interest that, in Th2 cell clones, differential sensitivity of IL-4 and IL-5 to CsA has been shown, linking differential regulation of Th2 cell cytokines to NFAT activation (Bohjanen et al., 1990; Naora et al., 1994). Our results suggest that cytokine production by Th17 cells, like the Th1 and Th2 effector cell lineages, is affected by the type and strength of TCR signals they receive. These findings open a new window in understanding the factors that control the expression of cytokines by this important lineage, which may be important for understanding therapeutic approaches to inflammatory and autoimmune diseases.

EXPERIMENTAL PROCEDURES

Mice

Itk$^{-/-}$ (Liao and Littman, 1995), *Rlk*$^{-/-}$*Itk*$^{-/-}$ (Schaeffer et al., 1999), and WT mice, backcrossed five generations on C57BL/6 background, were used between 7 and 9 weeks of age. Patterns of cytokine production were confirmed and in vivo challenges were performed with animals backcrossed to C57BL/6 mice for ten to 12 generations. Animal husbandry and experiments were performed in accordance with approved protocols by the National Human Genome Research Institute's Animal Use and Care Committee or the Office of Research Protection's Institutional Animal Care and Use Committee at Pennsylvania State University.

Isolation of Naive CD4$^+$ T Cells and Cell Culture

T cells were purified by T cell isolation columns (R&D) and then stained with anti-CD25-PE, anti-CD4-PerCPCy5.5, anti-CD8-APC, anti-CD44-FITC, and anti-CD62L-Pacific blue (eBioscience) and sorted on a FACsAria to obtain

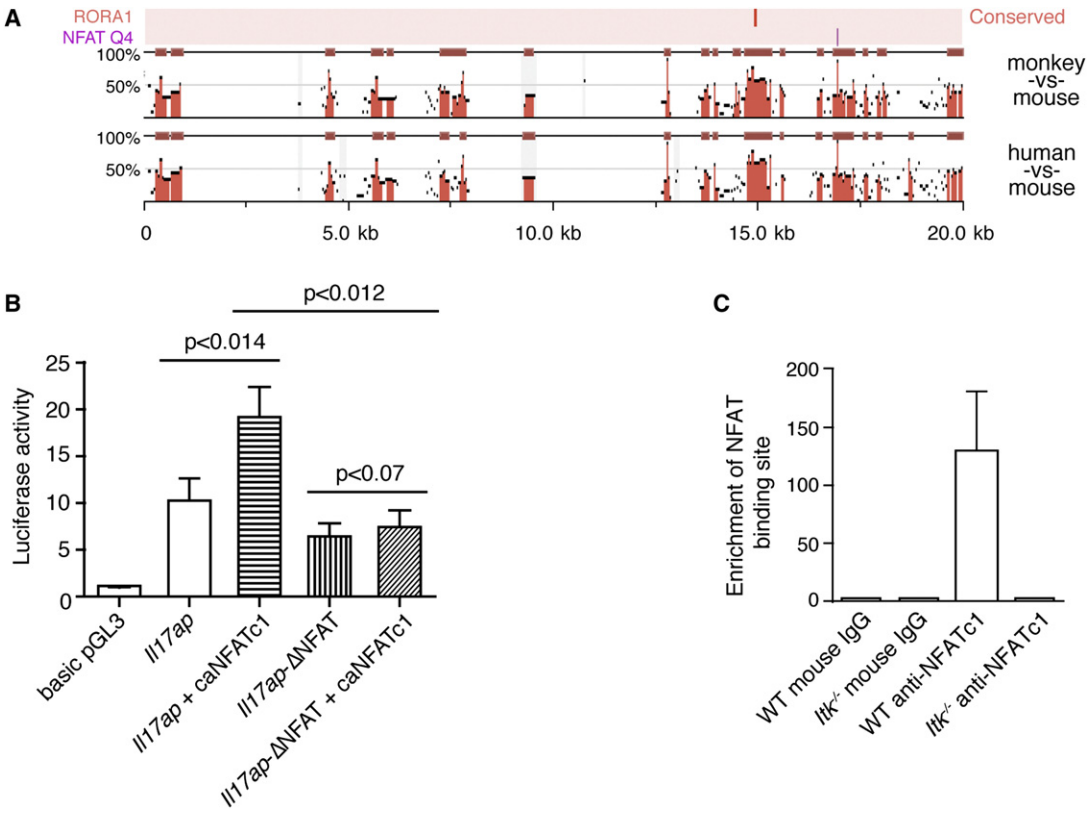


Figure 6. IL-17A Expression Is Linked to NFAT Activation

(A) Conserved NFAT and ROR binding sites (Yang et al., 2008b) across species in the 20 kb upstream of the *Il17a* gene, as predicted with the Mulan software at NCBI DCODE. See Figure S4A for sequence of the conserved NFAT binding site.

(B) Jurkat E6 cells were electroporated with *Il17ap* luciferase reporter construct (−3500 to +1 from mouse *Il17a* promoter subcloned in pGL3-basic) or with *Il17ap*-ΔNFAT (potential NFAT binding site −3085 to −3077 deleted) in the presence or absence of caNFATc1-pMIGR plasmid. After 24 hr, luciferase activity was quantified and normalized to Renilla luciferase. Results are expressed as fold increase in luciferase activity relative to pGL3-basic plasmid. Means ± SEM of triplicate samples from four experiments are shown. Statistics were calculated with the paired Student's t test.

(C) ChIP using NFATc1 antibody and amplifying the region around −3085 bp from the annotated first exon. Data are representative of duplicate experiments and were normalized to input value and expressed as fold enrichment relative to normal mouse sera.

naive $CD4^+CD44^{lo}CD62L^{hi}$ or $CD4^+CD44^{lo}CD62L^{hi}CD25^-$ at a purity greater than 99%. Similar results were obtained with either type of naive population.

Cells were cultured in RPMI 1640 or IMDM supplemented with 10% Fetal Calf Serum (Hyclone), 2 mM L-glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin, and 5 mM 2-B-mercaptoethanol (Invitrogen). Sorted naive $CD4^+$ T cells (2×10^5) were cocultured at a ratio of 1:5 with mitomycin-treated T depleted splenocytes as APCs for 1–4 days in 48-well plates containing 1 μg/ml of anti-CD3 (2C11) plus 3 μg/ml anti-CD28 (BioXcell) under different conditions: neutral conditions (Th0) used anti-IL-4, anti-IFN-γ, and anti-IL-12 antibodies (at 10 μg/ml); Th1 used 40 ng/ml IL-12 and anti-IL-4; Th17 used 20 ng/ml of IL-6, 5 ng/ml of TGF-β1, anti-IL-4, anti-IFN-γ, and anti-IL-12; IL-21 and TGF-β1 used 100 ng/ml of IL-21 plus 5 ng/ml of TGF-β1, anti-IL-4, anti-IFN-γ, and anti-IL-12; IL-6 used 20 ng/ml of IL-6 plus anti-IL-4, anti-IFN-γ, and anti-IL-12; and IL-1α/β and IL-6 used 100 ng/ml of either IL-1α or IL-1β plus 20 ng/ml IL-6, anti-IL-4, anti-IFN-γ, and anti-IL-12. IL-23 was used at 20 ng/ml. Cytokines were purchased from Peprotech, with the exception of TGF-β and IL-23, which were from R&D. Cytokine antibodies were from BioXcell. The calcineurin inhibitors CsA (Sigma) and FK506 (Sigma) were added to the naive CD4 T cells and APC cocultures for 30 min before stimulation with Th17 cell cocktails, and the cells were cultured for 48 hr. Cells were cultured with 1 μg/ml of anti-CD3 plus 1 μM ionomycin for 48 hr under Th17 cell conditions.

Cytokine Analyses

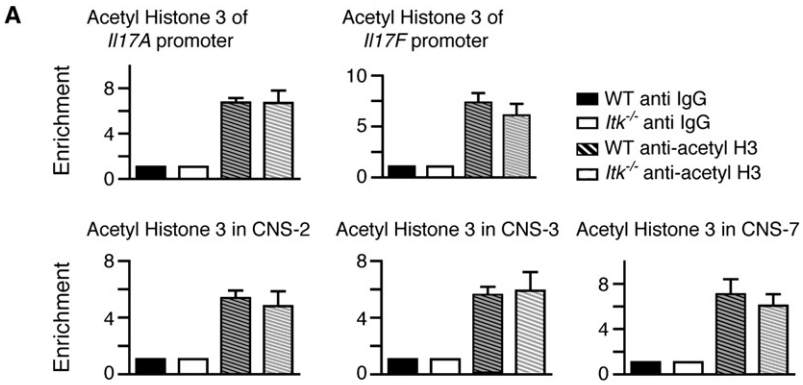
For intracellular staining, cells were differentiated for 2–3.5 days, then stimulated with 50 ng/ml of PMA (Sigma) and 1 μg/ml of ionomycin (Sigma) or with 1 μg/ml anti-CD3 and 3 μg/ml anti-CD28 in presence of golgi-plug for 4 hr. Intracellular cytokines were stained with anti-IL-17A, -IL-17F, -IFN-γ, or -FoxP3 (eBioscience). Data acquisition was done on an LSRII (BD Biosciences) and analyzed by FlowJo software (Tree Star). ELISAs were performed on supernatants from 48 hr cultures with Mouse IL-17A (eBioscience) and Duo Set IL-17F (R&D) ELISAs.

CFSE

$CD4^+CD25^-CD44^{lo}CD62L^{hi}$ (3×10^6 cells/ml) were incubated in PBS containing 2.5 μM of CFSE (Molecular Probes) for 10 min (room temperature), which was quenched by adding 1 ml of FBS for 1 min. Cells were washed twice with complete RPMI and stimulated for 3 days.

Reverse-Transcription Quantitative PCR

Total RNA was prepared from differentiated T cells at differing times after stimulation and from lungs from immunized and OVA-challenged WT and *Itk*$^{-/-}$ mice with Trizol reagent (Invitrogen) and RNeasy Mini kit (QIAGEN). cDNA was synthesized with the Taqman Reverse kit (Applied Biosystems). Quantitative RT-PCR was performed on a 7500 Fast Real-Time PCR instrument (Applied Biosystems) with either TaqMan Universal PCR Master Mix (Applied Biosystems) for *Il17a*, *Il17f*, *Il21*, *Il22*, and *Rorc* (Applied Biosystems) or Platinum SYBR Green qPCR Supermix (Invitrogen) for *Rora* (5′-TCTCCCTGCGCT CTCCGCAC-3′ and 5′-TCCACAGATCTTGCATGGA-3′). 18srRNA and *Thy1* were used for normalization for in vitro-differentiated $CD4^+$ T cells and for lungs, respectively (*Thy1* was used to normalize for T cells numbers in the



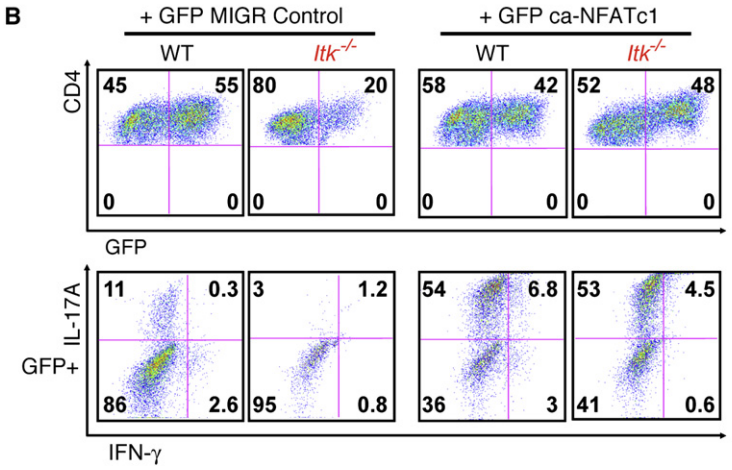


Figure 7. The IL-17 Locus Has an Open Chromatin Conformation, and caNFATc1 Rescues the IL-17A Defect in *Itk*$^{-/-}$ Cells

(A) ChIP using acetylated histone H3 antibody and amplifying regions just upstream of the transcriptional start sites and CNS 2, 3, and 7 in the *Il17* locus (see Figure S4C). Data were normalized to input value and expressed as fold enrichment relative to normal rabbit sera. Data are the mean ± SEM of four independent experiments.

(B) Production of IL-17A after retroviral transduction with an activated NFATc1 construct. Data are from one of three independent experiments.

lungs). The data in differentiated CD4$^+$ T cells was expressed relative to naive CD4$^+$ T cells; whereas mRNA expression in lungs from treated animals was expressed relative to a WT-challenged mouse. The data were expressed as $2^{-\Delta\Delta CT}$ with the ABI 7500 SDS 1.3.1 software.

Ovalbumin-Induced Airway Hypersensitivity

Mice were sensitized with ovalbumin (Sigma-Aldrich) complexed to aluminum hydroxide (10 μg ovalbumin/1 mg alum; Pierce) intraperitoneally in a total volume of 200 μl on days 0 and 5. Mice were later challenged intranasally with ovalbumin from days 12 through 15 (at a concentration of 2 mg/ml, for a total of 40 μg total exposure). Development of allergic asthma was confirmed by analyzing airway hyperresponsiveness (AHR) on day 16 with a custom-made mechanical ventilator as previously described. Mice were then sacrificed, and lungs were used for RNA or sectioned and stained by periodic acid-Schiff (PAS) as detailed (Ferrara et al., 2006; Mueller and August, 2003).

Chromatin Immunoprecipitation

ChIP assays were performed with EZ-Magna ChIP A (Upstate) as recommended by the manufacturer. In brief, after being fixed in 1% formaldehyde, T cells were lysed for 10 min at room temperature. Chromatin was sheared by sonication in Sonicator 3000 (Misonix). Lysates equivalent to 2 × 10^6 cells were used per immunoprecipitation at 4°C overnight with 5 μg of anti-NFATc1, (sc-7294, Santa Cruz Biotechnology), anti-acetylated Histone H3 (06-599B, Upstate), anti-trimethyl K4 Histone H3 (39159, Active Motif), or preimmune mouse or rabbit IgG antibodies. Enrichment of chromatin was analyzed with Platinum SYBR Green qPCR Supermix (Invitrogen) and the 7500 Fast Real-Time PCR instrument (Applied Biosystems). Data were normalized to input values and expressed as fold enrichment relative to normal mouse or rabbit sera.

The primer sequences used for qPCR analysis of the NFAT binding site in *Il17a* promoter are 5′-AATAGATTC TCAATGGTAGCC-3′ and 5′-GAAAATTCTTACTTTTGTA AACAG-3′. Primer sequences for CNS 2, 3, and 7 are indicated in (Akimzhanov et al., 2007). Conserved NFAT binding sites were found with Mulan software at the National Center for Biotechnology Information's DCODE.org.

Retrovirus Production and Infection

Constitutively active STAT3-IRES-GFP pMIGR (gift from D. Littman), ca-NFATc1-IRES-GFP-pMIGR (gift of N. Clipstone), and ITK-IRES-GFP-pMIGR (gift of L. Berg) plasmids (12.5 μg) were used to transfect 293 T cells with Fugene (Roche). After 48 hr, retroviral supernatants were collected.

Sorted naive CD4$^+$ T cells were cocultured with T depleted APCs under Th0 conditions for 48 hr. Retrovirus supernatants were added to the cells and spun at 2500 rpm for 1.5 hr at room temperature with 8 μg/ml of polybrene (Sigma). After 24 hr, infected cells were differentiated under Th17 cell conditions for 48 hr and stained for intracellular cytokines.

Immunoblot

After stimulation for the indicated times, 1 × 10^6 CD4 T cells were lysed in Laemmli buffer. Proteins were separated in 8% SDS-PAGE gel and transferred to nitrocellulose membranes, which were blocked and incubated with either anti-phospho-STAT3 or anti-STAT3 (9145L, 9132L, Cell Signaling Technology) as per the manufacturer's instructions, washed, incubated with horseradish peroxidase (HPR)-labeled goat anti-rabbit (Amersham Pharmacia), and developed with the enhanced chemiluminescence detection system (Amersham Pharmacia).

Luciferase Reporter Assays

DNA fragments corresponding to −3500 to +1 from mouse *Il17a* and *Il17f* promoters were subcloned into pGL3-basic (Promega) and designated as *Il17ap* or *Il17fp*. Plasmid *Il17ap*-ΔNFAT was constructed by deleting the potential NFAT binding site (−3085 to −3077) from *Il17ap* plasmid by overlapping PCR. Jurkat E6 cells (6 × 10^6) were electroporated with 20 μg luciferase reporter construct and 1 μg pRL-TK (Renilla luciferase) plasmid with or without 5 μg of caNFATc1-pMIGR plasmid by a BTX ECM 830 electroporator (BTX Technologies). After 24 hr, cells were lysed according to the manufacturer, and luciferase activity was quantified in triplicate in reaction mixtures containing 15 μl lysate and 100 μl reagent from Dual-Luciferase Reporter Assay System (Promega) with Lumat LB 9507 luminometer (Berthold Technologies). Each transfection was done in triplicate (or duplicate for Figure S4).

Statistical Analyses

Results were expressed as mean ± standard error of the mean (SEM). Statistical differences between the analyzed groups were calculated with the paired

Student's t test. Values of $p < 0.05$ are considered significant. Graphs were done in Excel (Microsoft) and Prism (GraphPad).

SUPPLEMENTAL DATA

Supplemental Data include four figures and can be found with this article online at http://www.cell.com/immunity/supplemental/S1074-7613(09)00413-0.

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REFERENCES

Akimzhanov, A.M., Yang, X.O., and Dong, C. (2007). Chromatin remodeling of interleukin-17 (IL-17)-IL-17F cytokine gene locus during inflammatory helper T cell differentiation. J. Biol. Chem. *282*, 5969–5972.

Berg, L.J. (2007). Signalling through TEC kinases regulates conventional versus innate CD8(+) T-cell development. Nat. Rev. Immunol. *7*, 479–485.

Berg, L.J., Finkelstein, L.D., Lucas, J.A., and Schwartzberg, P.L. (2005). Tec family kinases in T lymphocyte development and function. Annu. Rev. Immunol. *23*, 549–600.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L., and Kuchroo, V.K. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature *441*, 235–238.

Bettelli, E., Korn, T., and Kuchroo, V.K. (2007). Th17: The third member of the effector T cell trilogy. Curr. Opin. Immunol. *19*, 652–657.

Bohjanen, P.R., Okajima, M., and Hodes, R.J. (1990). Differential regulation of interleukin 4 and interleukin 5 gene expression: A comparison of T-cell gene induction by anti-CD3 antibody or by exogenous lymphokines. Proc. Natl. Acad. Sci. USA *87*, 5283–5287.

Brustle, A., Heink, S., Huber, M., Rosenplanter, C., Stadelmann, C., Yu, P., Arpaia, E., Mak, T.W., Kamradt, T., and Lohoff, M. (2007). The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. Nat. Immunol. *8*, 958–966.

Chen, W., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G., and Wahl, S.M. (2003). Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J. Exp. Med. *198*, 1875–1886.

Chen, Z., Laurence, A., Kanno, Y., Pacher-Zavisin, M., Zhu, B.M., Tato, C., Yoshimura, A., Hennighausen, L., and O'Shea, J.J. (2006). Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. Proc. Natl. Acad. Sci. USA *103*, 8137–8142.

Constant, S.L., and Bottomly, K. (1997). Induction of Th1 and Th2 CD4+ T cell responses: The alternative approaches. Annu. Rev. Immunol. *15*, 297–322.

Davidson, T.S., DiPaolo, R.J., Andersson, J., and Shevach, E.M. (2007). Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. J. Immunol. *178*, 4022–4026.

Dong, C. (2008). TH17 cells in development: An updated view of their molecular identity and genetic programming. Nat. Rev. Immunol. *8*, 337–348.

Ferrara, T.J., Mueller, C., Sahu, N., Ben-Jebria, A., and August, A. (2006). Reduced airway hyperresponsiveness and tracheal responses during allergic asthma in mice lacking tyrosine kinase inducible T-cell kinase. J. Allergy Clin. Immunol. *117*, 780–786.

Fowell, D.J., Shinkai, K., Liao, X.C., Beebe, A.M., Coffman, R.L., Littman, D.R., and Locksley, R.M. (1999). Impaired NFATc translocation and failure of Th2 development in Itk-deficient CD4+ T cells. Immunity *11*, 399–409.

Glimcher, L.H., and Murphy, K.M. (2000). Lineage commitment in the immune system: The T helper lymphocyte grows up. Genes Dev. *14*, 1693–1711.

Gomez-Rodriguez, J., Readinger, J.A., Viorritto, I.C., Mueller, K.L., Houghtling, R.A., and Schwartzberg, P.L. (2007). Tec kinases, actin, and cell adhesion. Immunol. Rev. *218*, 45–64.

Gwack, Y., Feske, S., Srikanth, S., Hogan, P.G., and Rao, A. (2007). Signalling to transcription: Store-operated Ca2+ entry and NFAT activation in lymphocytes. Cell Calcium *42*, 145–156.

Hermann-Kleiter, N., Gruber, T., Lutz-Nicoladoni, C., Thuille, N., Fresser, F., Labi, V., Schiefermeier, N., Warnecke, M., Huber, L., Villunger, A., et al. (2008). The nuclear orphan receptor NR2F6 suppresses lymphocyte activation and T helper 17-dependent autoimmunity. Immunity *29*, 205–216.

Hu, H., Djuretic, I., Sundrud, M.S., and Rao, A. (2007). Transcriptional partners in regulatory T cells: Foxp3, Runx and NFAT. Trends Immunol. *28*, 329–332.

Ishigame, H., Kakuta, S., Nagai, T., Kadoki, M., Nambu, A., Komiyama, Y., Fujikado, N., Tanahashi, Y., Akitsu, A., Kotaki, H., et al. (2009). Differential roles of interleukin-17A and −17F in host defense against mucoepithelial bacterial infection and allergic responses. Immunity *30*, 108–119.

Ivanov, I.I., McKenzie, B.S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J., and Littman, D.R. (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell *126*, 1121–1133.

Korn, T., Bettelli, E., Gao, W., Awasthi, A., Jager, A., Strom, T.B., Oukka, M., and Kuchroo, V.K. (2007). IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature *448*, 484–487.

Kouzarides, T. (2007). Chromatin modifications and their function. Cell *128*, 693–705.

Liao, X.C., and Littman, D.R. (1995). Altered T cell receptor signaling and disrupted T cell development in mice lacking Itk. Immunity *3*, 757–769.

Liu, K.Q., Bunnell, S.C., Gurniak, C.B., and Berg, L.J. (1998). T cell receptor-initiated calcium release is uncoupled from capacitative calcium entry in Itk-deficient T cells. J. Exp. Med. *187*, 1721–1727.

Liu, X.K., Clements, J.L., and Gaffen, S.L. (2005). Signaling through the murine T cell receptor induces IL-17 production in the absence of costimulation, IL-23 or dendritic cells. Mol. Cells *20*, 339–347.

Mangan, P.R., Harrington, L.E., O'Quinn, D.B., Helms, W.S., Bullard, D.C., Elson, C.O., Hatton, R.D., Wahl, S.M., Schoeb, T.R., and Weaver, C.T. (2006). Transforming growth factor-beta induces development of the T(H)17 lineage. Nature *441*, 231–234.

Mowen, K.A., and Glimcher, L.H. (2004). Signaling pathways in Th2 development. Immunol. Rev. *202*, 203–222.

Mueller, C., and August, A. (2003). Attenuation of immunological symptoms of allergic asthma in mice lacking the tyrosine kinase ITK. J. Immunol. *170*, 5056–5063.

Naora, H., Altin, J.G., and Young, I.G. (1994). TCR-dependent and -independent signaling mechanisms differentially regulate lymphokine gene expression in the murine T helper clone D10.G4.1. J. Immunol. *152*, 5691–5702.

Neal, J.W., and Clipstone, N.A. (2003). A constitutively active NFATc1 mutant induces a transformed phenotype in 3T3–L1 fibroblasts. J. Biol. Chem. *278*, 17246–17254.

Nurieva, R., Yang, X.O., Martinez, G., Zhang, Y., Panopoulos, A.D., Ma, L., Schluns, K., Tian, Q., Watowich, S.S., Jetten, A.M., and Dong, C. (2007a). Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature *448*, 480–483.

Nurieva, R.I., Chuvpilo, S., Wieder, E.D., Elkon, K.B., Locksley, R., Serfling, E., and Dong, C. (2007b). A costimulation-initiated signaling pathway regulates NFATc1 transcription in T lymphocytes. J. Immunol. *179*, 1096–1103.

Ouyang, W., Kolls, J.K., and Zheng, Y. (2008). The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity *28*, 454–467.

Schaeffer, E.M., Debnath, J., Yap, G., McVicar, D., Liao, X.C., Littman, D.R., Sher, A., Varmus, H.E., Lenardo, M.J., and Schwartzberg, P.L. (1999). Requirement for Tec kinases Rlk and Itk in T cell receptor signaling and immunity. Science *284*, 638–641.

Schaeffer, E.M., Yap, G.S., Lewis, C.M., Czar, M.J., McVicar, D.W., Cheever, A.W., Sher, A., and Schwartzberg, P.L. (2001). Mutation of Tec family kinases alters T helper cell differentiation. Nat. Immunol. *2*, 1183–1188.

Schwartzberg, P.L., Finkelstein, L.D., and Readinger, J.A. (2005). TEC-family kinases: Regulators of T-helper-cell differentiation. Nat. Rev. Immunol. *5*, 284–295.

Veldhoen, M., Hocking, R.J., Atkins, C.J., Locksley, R.M., and Stockinger, B. (2006). TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity *24*, 179–189.

Veldhoen, M., Hirota, K., Westendorf, A.M., Buer, J., Dumoutier, L., Renauld, J.C., and Stockinger, B. (2008). The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. Nature *453*, 106–109.

Veldhoen, M., Hirota, K., Christensen, J., O'Garra, A., and Stockinger, B. (2009). Natural agonists for aryl hydrocarbon receptor in culture medium are essential for optimal differentiation of Th17 T cells. J. Exp. Med. *206*, 43–49.

Weaver, C.T., Hatton, R.D., Mangan, P.R., and Harrington, L.E. (2007). IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annu. Rev. Immunol. *25*, 821–852.

Wei, L., Laurence, A., Elias, K.M., and O'Shea, J.J. (2007). IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. J. Biol. Chem. *282*, 34605–34610.

Winslow, M.M., Neilson, J.R., and Crabtree, G.R. (2003). Calcium signalling in lymphocytes. Curr. Opin. Immunol. *15*, 299–307.

Yang, X.O., Panopoulos, A.D., Nurieva, R., Chang, S.H., Wang, D., Watowich, S.S., and Dong, C. (2007). STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. J. Biol. Chem. *282*, 9358–9363.

Yang, X.O., Chang, S.H., Park, H., Nurieva, R., Shah, B., Acero, L., Wang, Y.H., Schluns, K.S., Broaddus, R.R., Zhu, Z., and Dong, C. (2008a). Regulation of inflammatory responses by IL-17F. J. Exp. Med. *205*, 1063–1075.

Yang, X.O., Pappu, B.P., Nurieva, R., Akimzhanov, A., Kang, H.S., Chung, Y., Ma, L., Shah, B., Panopoulos, A.D., Schluns, K.S., et al. (2008b). T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. Immunity *28*, 29–39.

Zhang, C., Zhang, J., Yang, B., and Wu, C. (2008a). Cyclosporin A inhibits the production of IL-17 by memory Th17 cells from healthy individuals and patients with rheumatoid arthritis. Cytokine *42*, 345–352.

Zhang, F., Meng, G., and Strober, W. (2008b). Interactions among the transcription factors Runx1, RORgammat and Foxp3 regulate the differentiation of interleukin 17-producing T cells. Nat. Immunol. *9*, 1297–1306.

Zhou, L., Ivanov, I.I., Spolski, R., Min, R., Shenderov, K., Egawa, T., Levy, D.E., Leonard, W.J., and Littman, D.R. (2007). IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat. Immunol. *8*, 967–974.

Zhou, L., Chong, M.M., and Littman, D.R. (2009). Plasticity of CD4(+) T Cell Lineage Differentiation. Immunity *30*, 646–655.

Zhu, J., and Paul, W.E. (2008). CD4 T cells: Fates, functions, and faults. Blood *112*, 1557–1569.